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STRUCTURE-ACTIVITY RELATIONSHIPS AND IMMUNOCHEMICAL STUDIES ON COBROTOXIN

by

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August 1971

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The carboxyl groups in cobrotoxin were modified with glycine methyl ester after activation with water-soluble carbodiimide. Six out of seven free carboxyls reacted in the absence of guanidine-HCl without altering the biological activity. When the remaining carboxyl was modified in the presence of 5 M guanidine-HCl, the resulting toxin was devoid of activity. This "buried" carboxyl is essential for activity and was identified as the γ-carboxyl group of Glu-21. (Author)

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ABSTRACT

Studies on The Status of Free Amino and Carboxyl Groups in Cobrotoxin

The two-dimensional structure of cobrotoxin has recently been established and permits a study of structure-activity relationships.. Preceding studies on the chemical modification of the single tryptophan and two tyrosyl residues in cobrotoxin suggested that either the intact tryptophan residue or the Tyr-25 is essential for full activity of the toxin.

Cobrotoxin is a basic protein having four free amino groups, one on N-terminal leucine and three on lysine residues at positions 26, 27 and 47. The status of free amino groups in cobrotoxin was studied by stepwise modification with trinitrobenzene sulfonate. Lys-27 was selectively modified without altering the activity of cobrotoxin. However, complete loss of the activity was observed when Lys-27 and Lys-47 were trinitrophenylated, suggesting that the ε-amino group of Lys-47 is essential for activity of cobrotoxin. The α-amino group of N-terminal leucine had not any correlation with activity was demonstrated by guanidination of the lysine residues with O-methylisourea followed by trinitrophenylation of the α-amino group.

Cobrotoxin contains seven free carboxyls, four on glutamyl and two on aspartyl residues, and one on C-terminal asparagine. The carboxyl groups in cobrotoxin were modified with glycine methyl ester after activation with water-soluble carbodiimide. Six out of seven free carboxyls reacted in the absence of guanidine-HCl without altering the biological activity. When the remaining carboxyl was modified in the presence of 5 M guanidine-HCl, the resulting toxin was devoid of activity. This "buried" carboxyl is essential for activity and was identified as the carboxyl group of Glu-21.

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Studies on The Status of Free Amino and Carboxyl Groups in Cobrotoxin

I. Introduction

The two-dimensional structure of cobrotoxin has recently been established by Yang et al. (1,2) and permits a study of structure-activity relationships. Preceding studies (3,4) on the chemical modification of the single tryptophan and two tyrosyl residues in cobrotoxin suggested that either the intact tryptophan residue or the Tyr-25 is essential for full activity of the toxin.

Cobrotoxin is a basic protein having four free amino groups, one on N-terminal leucine and three on lysine residues at positions 26, 27 and 47. In this communication, the status of free amino groups in cobrotoxin was studied by trinitrophenylation with trinitrobenzene sulfonate (TNBS). The reaction was reported as specific for free primary amino groups and can be followed spectrophotometrically (5-7). Cobrotoxin was also guanidinated with O-methylisourea followed by trinitrophenylation to elucidate the correlation of ε-amino group with the biological activity of the toxin.

Cobrotoxin contains seven free carboxyl groups, four on glutamyl and two on aspartyl residues, and one on C-terminal asparagine. In order to examine the importance of the free carboxyls for toxicity, the carboxyl groups were modified by the water-soluble carbodiimide-nucleophile procedure developed by Koshland Jr. et al. (8,9)

From the results of this investigation, the amino and carboxyl groups which are essential for the biological functions of cobrotoxin have been differentiated, and the positions of these groups in the sequence of amino acid have also been established.

II. Materials and Methods

Cobrotoxin was prepared from Taiwan cobra (Naja naja atra) venom as previously described (10). TNBS was prepared from a commercial product of the sodium salt (Tokyo Kasei Co.) as follows: the preparation was dissolved in 1 N HCl to make 5 % solution and the intensely orange-colored solution was decolorized by passing through a column of Norit-Celite (1 : 1, by wt.). By concentrating the filtrate in vacuo, TNBS was obtained as almost colorless crystal. ε-TNP-lysine was synthesized essentially according to the method of Okuyama and Satake (5), except that instead of picryl chloride TNBS was used. The product gave a single yellow spot (R_f=0.40) on descending paper chromatogram in

iso-amyl alcohol : n-butanol : ethanol : 0.1 M phthalate buffer, pH 6.0 (30 : 30 : 11 : 45, by vol.) and gave a positive ninhydrin test. 1-Ethyl-3-dimethylaminopropyl carbodiimide-HCl (EDC-HCl) was contributed from the laboratory of Dr. Koshland, University of California. The ^{14}C-glycine methyl ester-HCl was prepared from glycine-2-^{14}C (New England Nuclear) by esterification in HCl-saturated methanol and diluted with unlabeled glycine methyl ester-HCl. O-methylisourea-HCl was purchased from Nutritional Biochemicals Corp. Trypsin and chymotrypsin were the products of Worthington Biochemical Corp. Reagent grade glycine methyl ester-HCl, urea, guanidine-HCl and iodoacetic acid were purchased from Nakarai Chemicals, Ltd. All other reagents were of analytical grade. Urea and iodoacetic acid were crystallized before use.

1. Trinitrophenylation of cobrotoxin with TNBS

Trinitrophenylation of free amino groups was performed essentially according to the method of Habeeb (11). Cobrotoxin solution (2 mg/ml of 4 % $NaHCO_3$, pH 8.5) in a series of tubes were mixed with 0.5 ml of 0.3 % TNBS and the reaction was allowed to proceed at 37°. After varying intervals of time, the reaction was stopped by the addition of 0.5 ml of 0.4 N HCl. The absorbance at 345 mu was measured after dilution with 0.1 N HCl and the number of amino groups trinitrophenylated was calculated according to the method of Habeeb (11).

2. Stepwise modification of amino group with TNBS

In order to differentiate the "essential" amino group(s) for biological activity, cobrotoxin was reacted with 1.1 and 2.2-fold molar excess of TNBS, respectively. The reaction was carried out in 0.1 M borate buffer (pH 8.6) for 1 h and the product was chromatographyed on a column of DEAE-cellulose by stepwise elution. The main protein peak was lyophilized and desalted by passage through a column of Sephadex G-25. The protein fractions were then pooled and lyophilized.

For determination of the position of the "essential" amino group(s) in the sequence of amino acid in cobrotoxin, the TNP-cobrotoxin was reduced and S-carboxymethylated (RCM-) under the procedure described by Crestfield *et al.* (12) followed by tryptic digestion. The RCM-TNP-cobrotoxin was dissolved in 0.1 M NH_4HCO_3 buffer (pH 8.0) to make a 0.5 % solution and trypsin (50 : 1) was added. Digestion was carried out at 37° for 4 h and the digest was lyophilized.

TNP-peptide from the tryptic digest was separated by a combination of high voltage paper electrophoresis at pH 3.6 with pyridine-acetic acid-water (1 : 10 : 89, by vol.) and descending

paper chromatography with n-butanol-acetic acid-water-pyridine (15 : 3 :12 : 10, by vol.) as previously described (4). TNP-peptide was detected as a yellow-colored spot on the paper and the isolated peptide was proved to be electrophoretically homogeneous.

3. Guanidination of cobrotoxin with O-methylisourea

The guanidination of proteins by means of O-methylisourea generally leads to selective modification of the ε-amino groups in lysine residues, while the reaction does not affect the α-amino group (13). In order to elucidate the correlation of the α-amino group of N-terminal leucine to the biological activity, cobrotoxin was first guanidinated with O-methylisourea followed by trinitrophenylation with TNBS. Guanidination was performed essentially according to the method of Chervenka and Wilcox (13). Cobrotoxin (3 μ moles) in 2 ml of 0.5 M O-methylisourea-HCl solution was adjusted to pH 10.8 with 6 N NaOH and the reaction was proceeded at 4° for 72 h. The mixture was then passed through a column of Sephadex G-25, and the protein fractions were pooled and lyophilized.

4. Modification of carboxyl groups

The modification of carboxyl groups in cobrotoxin was performed essentially according to the method of Hoare and Koshland (8).

a. Modification with EDC-HCl and glycine methyl ester (partially carboxy (6-COOH)-modified cobrotoxin): Cobrotoxin (60 mg) was dissolved in 3 ml of 1.0 M glycine methyl ester-HCl. The pH was adjusted to 4.75 and immediately the reaction initiated by the addition of solid EDC-HCl to a concentration of 0.2 M. The pH was maintained at 4.75 by the addition of 1 N HCl and the reaction was allowed to proceed at room temperature for 3 h. Excess reagents were then removed by passage through a column of Sephadex G-25, equilibrated with 1 % acetic acid, and the protein fraction was lyophilized. As a result of this experiment, six out of seven free carboxyl groups in cobrotoxin were modified in the absence of guanidine-HCl. The resulting 6-COOH-modified toxin showed a single band on disc electrophoresis and the results of amino acid analysis are shown in Table III.

b. Modification with EDC-HCl and glycine methyl ester in the presence of 5 M guanidine-HCl (completely carboxy (7-COOH)-modified cobrotoxin): Cobrotoxin (10 mg/ml) and glycine methyl ester-HCl (1.0 M) were dissolved in 5 M guanidine-HCl and the solution adjusted to pH 4.75 and reacted with solid EDC-HCl as described above. The modified toxin was homogeneous by the criteria of disc electrophoresis and the amino acid composition shown in Table III.

c. Modification of 6-COOH-modified cobrotoxin with EDC-HCl and ^{14}C-glycine methyl ester-HCl in the presence of 5 M guanidine-HCl: 6-COOH-modified cobrotoxin (50 mg/2 ml) and ^{14}C-glycine methyl ester-HCl (1.0 M) were dissolved in 5 M guanidine-HCl. The subsequent procedures were performed as described above.

5. Determination of the position of the "essential" carboxyl group in the sequence of amino acid in cobrotoxin

^{14}C-labeled cobrotoxin (30 mg) was reduced and S-carboxymethylated under the procedure described by Crestfield et al. (12) followed by tryptic digestion as described above. For isolation of "^{14}C-T-peptide", the digest was fractionated by high voltage paper electrophoresis at pH 3.6 in pyridine-acetic acid-water (1 : 10 : 89, by vol.) on a strip of Toyo Roshi No. 50 at 35 v/cm for 90 min. The paper was then cut into 2-cm strips and the radioactivity was measured with a Aloka model PSC-4 gas flow counter. The radioactive fraction was eluted with 1 % acetic acid and further purified by descending paper chromatography with n-butanol-pyridine-acetic acid-water (15 : 10 : 3 : 12, by vol.). The "^{14}C-T-peptide" eluted was hydrolyzed with 6 N HCl at 105° for 24 h and subjected to amino acid analysis. The amino acid composition showed that the peptide was derived from residues Leu(1) - Lys(26) of cobrotoxin.

The "^{14}C-T-peptide" (Ca. 3 mg) obtained from tryptic digest was dissolved in 0.25 ml of 0.2 M NH_4HCO_3 buffer (pH 8.1) and 0.05 ml of 1 % chymotrypsin solution in the same buffer was added. The mixture was kept at 37° for 16 h and the hydrolysate was worked up as described above. The "^{14}C-C-peptide" was subjected to Edman degradation and amino acid analysis.

Amino acid analysis was performed according to the procedure of Spackman et al. (14) with a Hitachi model KLA-3B automatic amino acid analyzer. Samples were hydrolyzed with 6 N HCl at 105° for 24 h. The sequence of amino acid residues in the isolated peptides was determined primarily by the Edman PTH procedure as previously described (1). PTH-amino acids were identified by thin layer chromatography.

Polyacrylamide gel electrophoresis, measurements of lethal toxicity and immunological procedures performed were essentially the same as previously described (4).

III. Results

1. Absorption spectrum of TNP-cobrotoxin

As shown in Fig. 1, the absorption spectrum of fully trinitrophenylated cobrotoxin which showed a maximum near 345 mμ and a flat at 410-420 mμ was practically the same as those of TNP-amino acids and TNP-peptides reported by Okuyama and Satake (5) and Satake et al. (6) The molar extinction coefficient (ε) of TNP-cobrotoxin at 345 mμ was found to be 4.76×10^4, i. e., 1.19×10^4 per mole of amino group. This value is very close to those of TNP-ε-lysine and TNP-α-amino group of amino acid and peptides (5,6). The result indicates that four equivalents of TNP-group were introduced into the cobrotoxin molecule.

2. Trinitrophenylation of cobrotoxin

As shown in Fig. 2, the reaction velocity of trinitrophenylation of cobrotoxin was markedly influenced by pH of the reaction mixture. The reaction proceeds more rapidly in an alkaline solution and completes rapidly. However, at near neutral pH the reaction proceeds very slowly.

The relation between the extent of modification and the lethal toxicity of cobrotoxin is shown in Fig. 3. It can be seen that the lethality decreased rapidly as trinitrophenylation proceeds. The lethality lost almost completely when two or more amino groups were modified.

3. Status of amino groups in cobrotoxin

In order to differentiate the "essential" amino group(s) for activity of cobrotoxin, the toxin was initially allowed to react with 1.1-fold molar excess of TNBS, and the product was purified by DEAE-cellulose chromatography with stepwise elution. An electrophoretogram of the tryptic digest of BCM-TNP-cobrotoxin revealed only one yellow-colored spot. The spot which represents the TNP-NH_2 containing peptide was eluted, purified by descending paper chromatography and hydrolyzed for amino acid analysis and paper chromatography. Although only one amino acid, arginine, was detected in amino acid analysis, a yellow-colored spot corresponding to a synthetic ε-TNP-lysine and another Sakaguchi test positive spot were detected by descending paper chromatography in the solvent system iso-amyl alcohol-n-butanol-ethanol-0.1 M phthalate buffer, pH 6.0 (30 : 30 : 11 : 45, by vol.). The result indicates that the peptide represents the sequence of ε-TNP-Lys-Arg located at positions 27 and 28 of cobrotoxin since these two amino acids do not occur together elsewhere in the molecule (Fig. 4A). This finding suggestes that Lys-27 in cobrotoxin is most accessible to trinitrophenylation with TNBS.

Cobrotoxin was further allowed to react with 2.2-fold molar excess of TNBS and the product was purified as described above. An electrophoretogram of the tryptic digest of RCM-TNP-cobrotoxin revealed two yellow-colored spots which represent the TNP-NH_2 containing peptides were eluted, purified by descending paper chromatography and hydrolyzed for amino acid analysis and paper chromatography. One of the two peptides gave the same result as above which represents the sequence of ε-TNP-Lys-Arg and the other peptide gave the following amino composition: $Arg_{0.9}$, $CM\text{-}Cys_{3.8}$, $Asp_{2.9}$, $Thr_{1.8}$, $Ser_{0.3}$, $Glu_{0.9}$, $Pro_{1.0}$, $Gly_{3.1}$, $Val_{1.0}$, $Ile_{1.9}$. The amino acid composition showed that the peptide was derived from residues Gly(40) - Arg(59) of cobrotoxin (Fig. 4A), indicating that both ε-amino groups of Lys-27 and Lys-47 were trinitrophenylated under the experimental conditions.

4. Characterization of Lys-27 TNP-cobrotoxin and Lys-27 & 47 TNP-cobrotoxin

As shown in Fig. 5, both TNP-cobrotoxins were revealed as a single band on polyacrylamide gel electrophoretogram. Lys-27 TNP-cobrotoxin migrated more slowly toward cathod than cobrotoxin, and Lys-27 & 47 TNP-cobrotoxin migrated toward the opposite side. No unreacted cobrotoxin was found in either TNP-derivatives, indicating that both preparations are electrophoretically homogenous.

As presented in Table I, complete loss of lethality was observed when both ε-amino groups of Lys-27 and Lys-47 were trinitrophenylated. However, the lethality of Lys-27 TNP-cobrotoxin remained unchanged, suggesting that the ε-amino group of Lys-27 which is most accessible to trinitrophenylation is not essential for lethal toxicity of the toxin.

As illustrated in Fig. 6, the Lys-27 TNP-cobrotoxin gave a precipitin line of identity with cobrotoxin, while Lys-27 & 47 TNP-cobrotoxin gave none at all on immunodiffusion in agar gel with anti-cobrotoxin sera.

As shown in Fig. 7, Lys-27 TNP-cobrotoxin gave almost the same maximal precipitation as cobrotoxin by quantitative precipitin reactions, while Lys-27 & 47 TNP-cobrotoxin gave almost no precipitate. This indicates that the ε-amino group of Lys-47 is essential for the biological activity of cobrotoxin.

5. Modification of α-amino group

The result of guanidination of cobrotoxin with O-methylisourea showed that essentially all three lysine residues were converted to homoarginine (Table II). The α-amino group of the terminal leucine did not react. The guanidinated cobrotoxin which

retained only one free α-amino group was further trinitrophenylated with TNBS. The lethal toxicity of the guanidinated cobrotoxin and of the TNP-guanidinated derivative remained unchanged (Table II), suggesting that the α-amino group of terminal leucine had no correlation with the lethal toxicity of the toxin.

The antigenic activity of these preparations was also studied with anti-cobrotoxin sera. Either guanidinated cobrotoxin or TNP-guanidinated toxin showed almost the same precipitin line as that of cobrotoxin on immunodiffusion in agar gel (Fig. 6) and precipitates by quantitative precipitin reactions (Fig. 8). The results indicate that the α-amino group of cobrotoxin is not involved in the biological activity of the toxin.

6. Characterization of carboxy-modified cobrotoxin

The number of modified carboxyl group is determined by analysis for incorporated glycine. The results of amino acid analysis of modified derivatives (Table III) showed that six of the seven carboxyl groups were modified, while the remaining carboxyl group reacted in the presence of 5 M guanidine-HCl. This indicats that one of the seven carboxyl groups in cobrotoxin is buried in the molecule, thus becoming unreactive in the absence of guanidine-HCl. No other amino acid residues in cobrotoxin were modified.

Since much deduction of the negative charge of carboxyl groups after modification, both modified toxins migrated further toward cathod than did cobrotoxin (Fig. 5) and revealed electrophoretically as a single band. No unreacted cobrotoxin was found in either modified preparations, indicating that complete modification had occurred under the experimental conditions.

7. Biological activity of carboxy-modified cobrotoxin

The effect of chemical modification of carboxyl groups on lethal toxicity of cobrotoxin is given in Table IV. The toxin with six modified carboxyls showed little effect on the lethality, while modification of all seven carboxyls led to complete loss of lethality, suggesting that one carboxyl group which is not accessible to modification in the absence of guanidine-HCl is essential for the toxicity of cobrotoxin.

As illustrated in Fig. 9, the six carboxy-modified cobrotoxin gave a precipitin line of identity with cobrotoxin, while the completely carboxy-modified preparation gave none at all as tested on immunodiffusion in agar gel with anti-cobrotoxin sera.

A slight decrease of antigenic activity was observed on six carboxy-modified cobrotoxin as measured by quantitative precipitin

reactions (Fig. 10). However, the completely carboxy-modified preparation gave almost no precipitate.

8. Identification of the "buried" carboxyl group essential for the biological activity of cobrotoxin

In order to identify the "buried" carboxyl group which is essential for the biological activity of cobrotoxin, the "^{14}C-C-peptide" isolated by the procedure described in Methods was subjected to amino acid analysis and Edman degradation. The "^{14}C-C-peptide" gave the following amino acid composition: CM-$Cys_{0.8}$, $Asp_{1.4}$, $Thr_{1.0}$, $Ser_{0.7}$, $Glu_{1.0}$, $Gly_{3.5}$, and the N-terminal amino acid of the peptide was determined by Edman degradation as glycine. The remaining peptide, after one cycle of degradation, was subjected to amino acid analysis and gave the following composition: CM-$Cys_{0.8}$, $Asp_{1.2}$, $Thr_{1.0}$, $Ser_{1.0}$, $Glu_{1.0}$, $Gly_{2.5}$. The results indicate that the "^{14}C-C-peptide" was shown to be the peptide of residues Gly-16 to Asn-23 (Fig. 4B). Referring to these results, it was evident that the carboxyl group in buried state and essential for the biological activity of cobrotoxin is the γ-carboxyl group of Glu-21.

IV. Discussion

There are four free amino groups in cobrotoxin. All four amino groups could be trinitrophenylated quantitatively by reaction with TNBS resulting in complete loss of the biological activity. TNBS was shown by Habeeb (11) to be a specific reagent for determining spectrophotometrically the number of free amino groups in proteins. The procedure provids a valuable tool for studying the effect of modification on the lethality of cobrotoxin. Complete loss of lethality was observed when more then two amino groups were modified indicating that of the four amino groups in cobrotoxin one or more might be essential for toxic action. In order to identify the "essential" amino group(s) stepwise modification with TNBS was carried out. Cobrotoxin was reacted first with 1.1-fold molar excess of TNBS. ε-Amino group of Lys-27 was the most accessible to trinitrophenylation but the biological activity unchanged, suggesting that the Lys-27 is not essential for the activity of the toxin. However, complete loss of biological activity was observed when Lys-27 and Lys-47 were modified with 2.2-fold molar excess of TNBS. These results indicate that the ε-amino group of Lys-47 is essential for the full activity of the toxin.

The guanidination of cobrotoxin resulted in that all lysine residues were converted to homoarginine without modification of

the α-amino group. The reaction revealed no effect on the biological activity of cobrotoxin.

The above results indicate that the effects of the two specific reagents on the biochemical and biological properties of cobrotoxin are different. Trinitrophenylation of cobrotoxin converts the positively charged amino group into neutral state while guanidination yields a substituted group that maintains the positive charge. Therefore, it is concluded that the positive charges contributed by the ε-amino groups of lysine residues in cobrotoxin may play important roles in the structural features for biochemical functions of the toxin.

The active guanidinated cobrotoxin which retains the only free α-amino group might be a valuable tool for determining the importance of the α-amine group for activity of the toxin. Trinitrophenylation of the guanidinated cobrotoxin with TNBS did not alter the biological activity, indicating that the α-amine group of N-terminal leucine is not essential for the activity of cobrotoxin.

There are seven free carboxyl groups in cobrotoxin. Among the reagents introduced for the modification of carboxylic acid side chains of proteins the activation by carbodiimide and attachment of nucleophiles (8,9) appears to be sufficiently specific. The number of modified carboxyl group is determined by analysis for incorporated glycine.

In native cobrotoxin six of the seven carboxyl groups were modified, while the remaining one reacted in the presence of 5 M guanidine-HCl. The six carboxy-modified toxin showed only a little loss in activity, while modification of all seven carboxyls led to complete loss of toxicity. In order to identify the "buried" carboxyl group, cobrotoxin was treated first with non-radioactive glycine methyl ester in the absence of guanidine-HCl, and was then incubated with ^{14}C-glycine methyl ester and carbodiimide in the presence of 5 M guanidine-HCl (15). The modified toxin containing ^{14}C-glycine was reduced and S-carboxymethylated. The alkylated protein was digested with trypsin to permit chromatographic isolation of a ^{14}C-labeled peptide, "^{14}C-T-peptide". The amino acid analysis revealed that the peptide was derived from residues Leu-1 to Lys-26. The "^{14}C-T-peptide" contains two free carboxyl groups in Glu-2 and Glu-21. Thus, to determine which free carboxyl group existed in the buried state, the "^{14}C-T-peptide" was digested with chymotrypsin and a radioactive peptide was separated by a combination of high voltage paper electrophoresis and descending paper chromatography. The radioactive peptide obtained was shown to be the peptide of residues Gly-16 to Asn-23. This was confirmed by determination of the N-terminal amino acid residue by Edman degradation.

Accordingly, the "buried" carboxyl group in cobrotoxin is on Glu-21 in the sequence of Gly-Cys-Ser-Gly-Gly-Glu(21)-Thr-Asn, and this carboxyl group is essential for the biological activity of cobrotoxin.

V. Conclusion

The status of free amino groups in cobrotoxin was studied by stepwise modification with trinitrobenzene sulfonate. Lys-27 was selectively modified without altering the activity of cobrotoxin. However, complete loss of the activity was observed when Lys-27 and Lys-47 were trinitrophenylated, suggesting that the ε-amino group of Lys-47 is essential for the activity of cobrotoxin. The α-amino group of N-terminal leucine had not any correlation with activity was demonstrated by guanidination of the lysine residues with O-methylisourea followed by trinitrophenylation of the α-amino group.

The carboxyl groups in cobrotoxin were modified with glycine methyl ester after activation with water-soluble carbodiimide. Six out of seven free carboxyls reacted in the absence of guanidine-HCl without altering the biological activity. When the remaining carboxyl was modified in the presence of 5 M guanidine-HCl, the resulting toxin was devoid of activity. This "buried" carboxyl is essential for activity and was identified as the β-carboxyl group of Glu-21.

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APPENDIX A-1

Table I

Effect of trinitrophenylation on lethal toxicity of cobrotoxin

	Number of $TNP-NH_2$	Lethality (%)
Cobrotoxin	0	100
Lys-27 TNP-cobrotoxin*	1	100
Lys-27 & 47 TNP-cobrotoxin**	2	0

Trinitrophenylation was carried out in 0.1 M borate buffer (pH 8.6) with 1.1-fold* and 2.2-fold** molar excess of TNBS, respectively, at room temperature (25°) for 1 h. The product was purified by chromatography on a column of DEAE-cellulose with stepwise elution.

APPENDIX A-2

Table II

Modification of α-amino group of cobrotoxin

	Amino acid residues found			Lethality (%)
	Leucine	Lysine	Homoarginine	
Cobrotoxin	1	3	0	100
Guanidinated Cobrotoxin	0.81	0	2.7	100
TNP-guanidinated Cobrotoxin*	0	0	2.7	100

* Trinitrophenylation of the guanidinated cobrotoxin was carried out in 0.1 M borate buffer (pH 8.6) with 10-fold molar excess of TNBS.

APPENDIX A-3

Table III

Amino acid composition of cobrotoxin and carboxy-modified derivatives

Amino acid	Residues per mole of protein		
		Modified derivatives	
	Cobrotoxin	In H_2O (pH 4.75)	In 5 M guanidine-HCl
Aspartic acid	8	8.0	8.3
Threonine	8	8.1	8.1
Serine	4	4.1	4.0
Glutamic acid	7	7.2	7.4
Proline	2	1.8	1.9
Glycine	7	13.1	13.9
Alanine	-	-	-
Half-cystine	8	7.9	8.0
Valine	1	1.1	1.1
Methionine	-	-	-
Isoleucine	2	2.0*	2.0
Leucine	1	0.92	0.94
Tyrosine	2	1.9	1.9
Phenylalanine	-	-	-
Lysine	3	2.9	2.9
Histidine	2	1.9	1.9
Arginine	6	6.0	6.0
Tryptophan	1	1.0	1.0

* All values in modified derivatives are expressed as molar ratios based on isoleucine=2.0.

APPENDIX A-4

Table IV

Modification of carboxyl groups in cobrotoxin with glycine methyl ester after activation by soluble carbodiimide

	Modified carboxyl groups	Lethality (%)
Cobrotoxin	0	100
In H_2O (pH 4.75)	6	75
In 5 M Guanidine-HCl	7	0

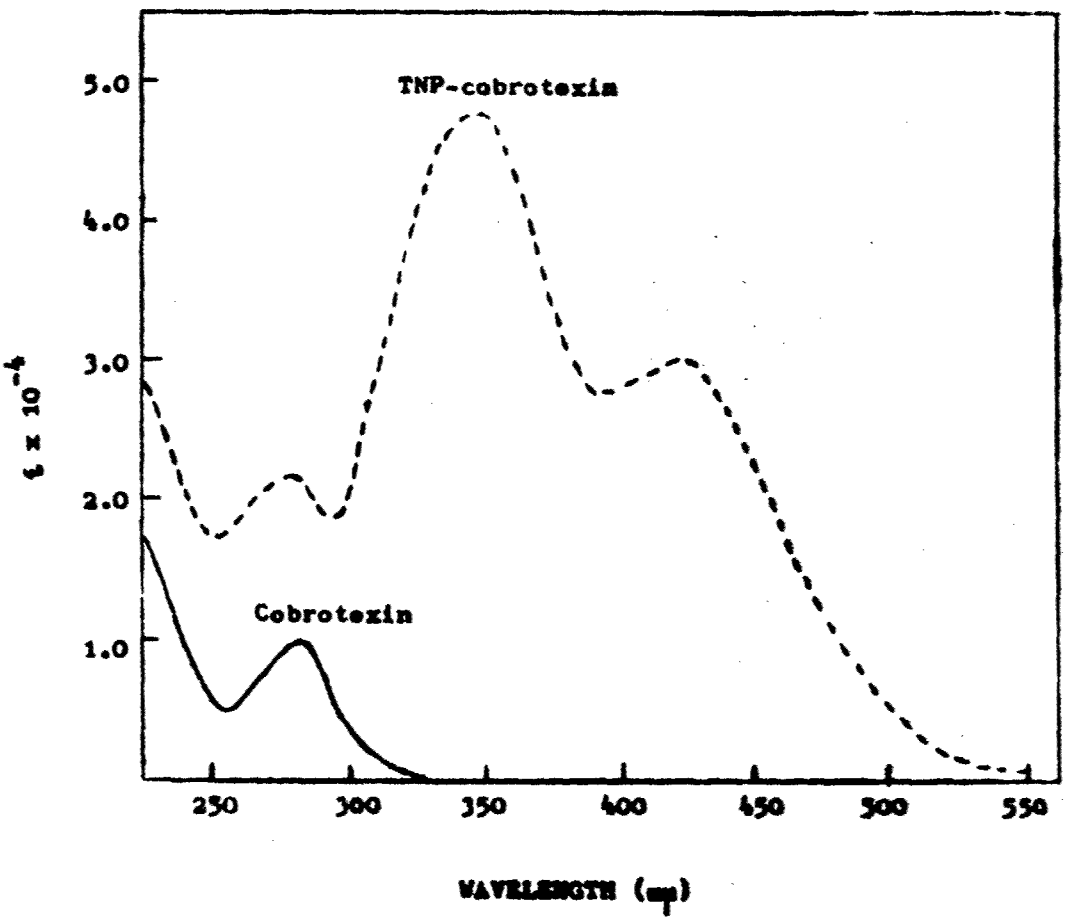


Fig. 1. Absorption spectra of cobrotoxin and fully trinitrophenylated cobrotoxin.

Absorption spectra of cobrotoxin were measured in 0.1 M phosphate buffer (pH 7.0) and TNP-cobrotoxin in 0.1 N HCl.

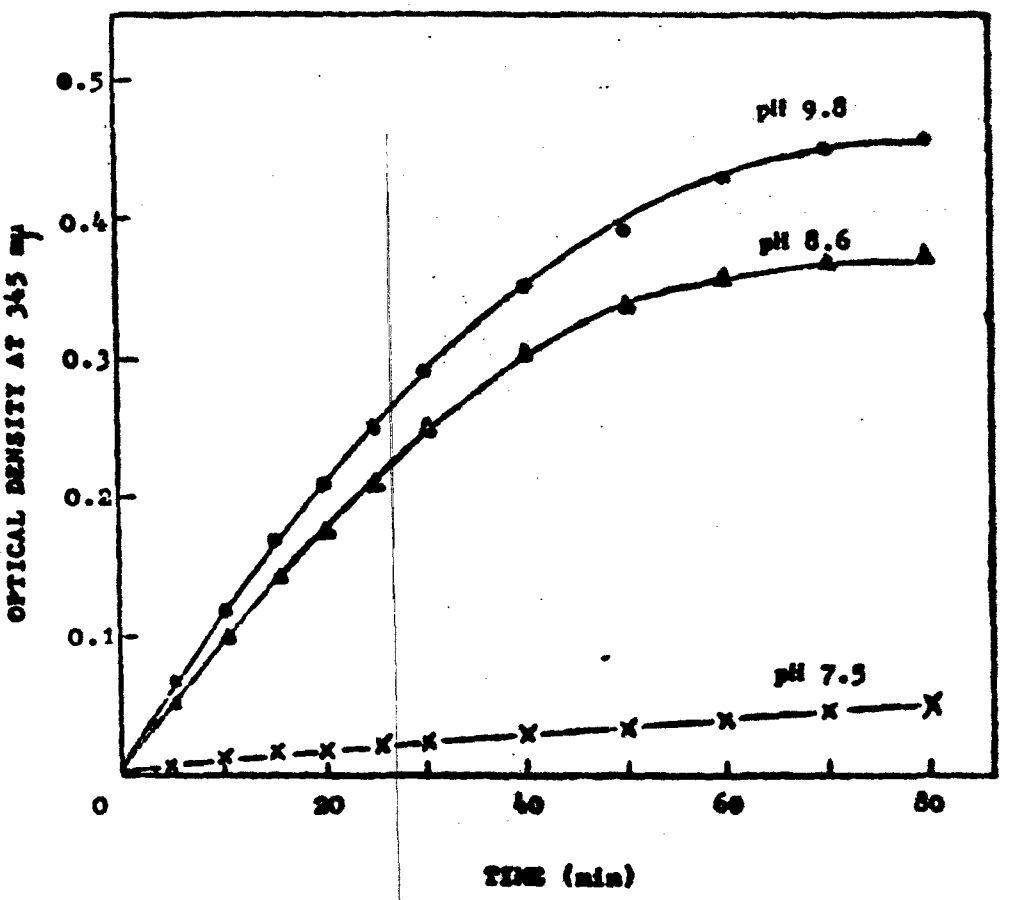


Fig. 2. Effect of pH on the trinitrophenylation of cobrotoxin.

Trinitrophenylation of cobrotoxin was carried out at room temperature (25°) in 1-cm quartz spectrophometer cell. 1 mg of cobrotoxin was dissolved in 3 ml of buffer solution and the reaction was initiated by the addition of 10-fold molar excess of TNBS. At suitable intervals of time, the optical density at 345 mμ was recorded. o——o, 4 % $NaHCO_3$ (pH 9.8); Δ——Δ, 0.1 M borate buffer (pH 8.6); X——X, 0.1 M phosphate buffer (pH 7.5).

APPENDIX B-3

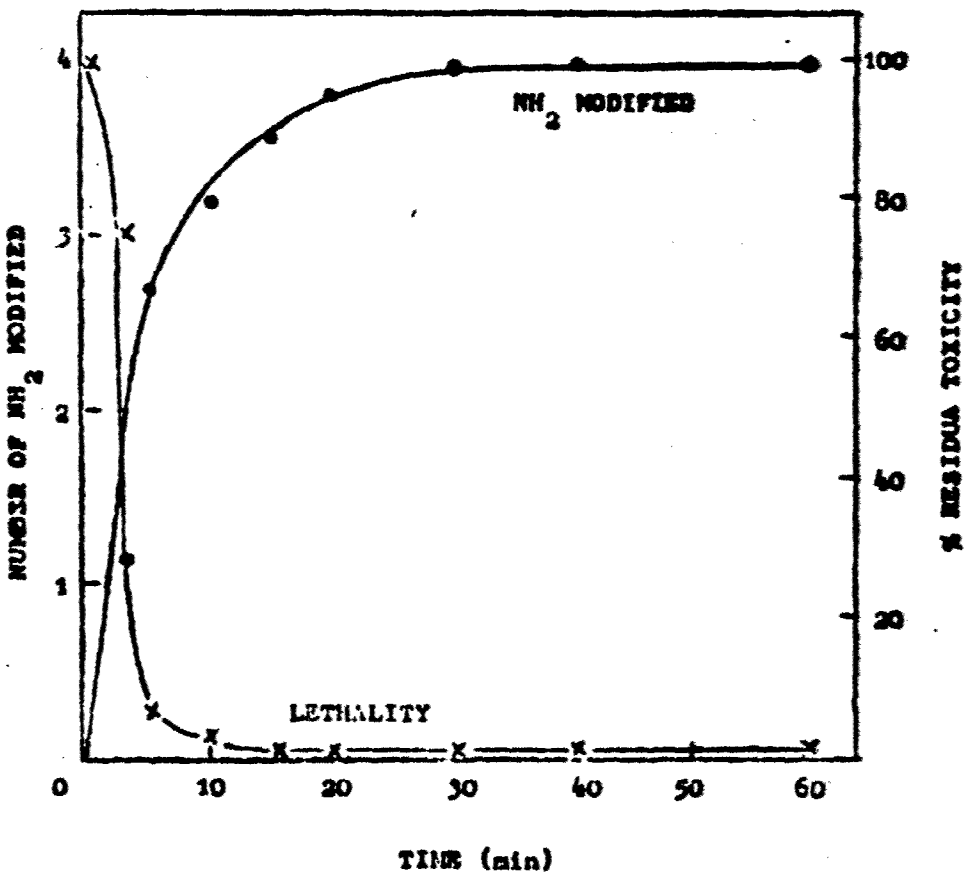
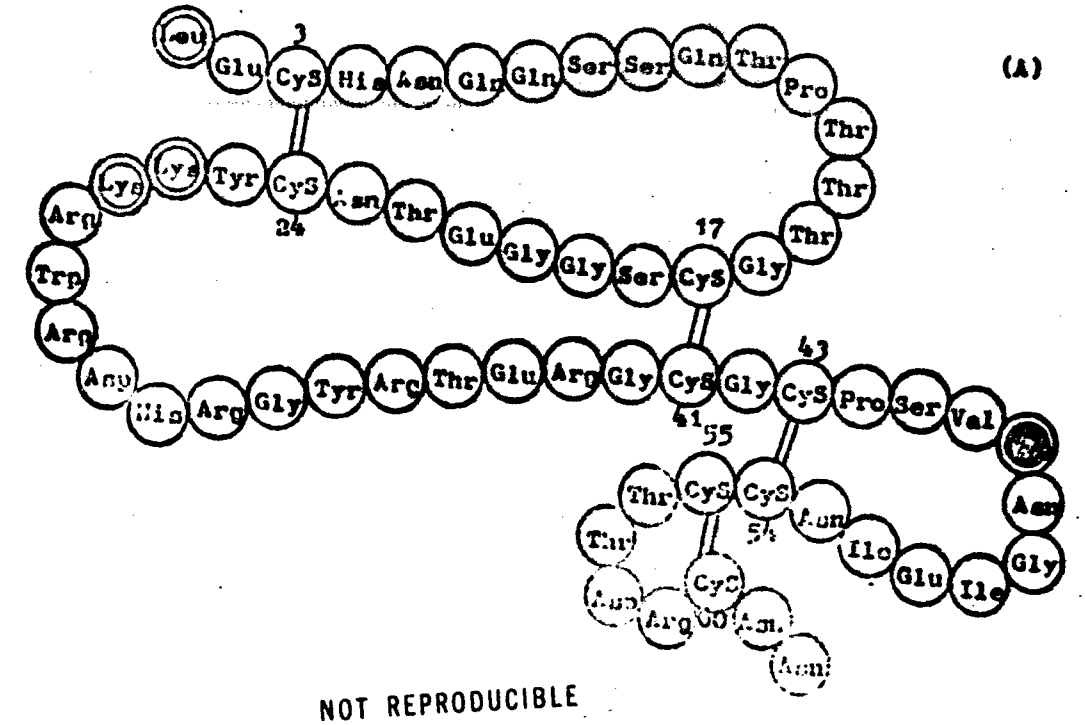


Fig. 3. Trinitrophenylation of free amino groups and decrease of lethal toxicity.

2 mg of cobrotoxin was dissolved in 1 ml of 4 % $NaHCO_3$ (pH 8.5) and 0.5 ml of 0.3 % TNBS in H_2O was added. Reaction was allowed to proceed at 37°. After suitable intervals of time, aliquots were taken for determination of lethality and the absorbancy at 345 mμ.



NOT REPRODUCIBLE

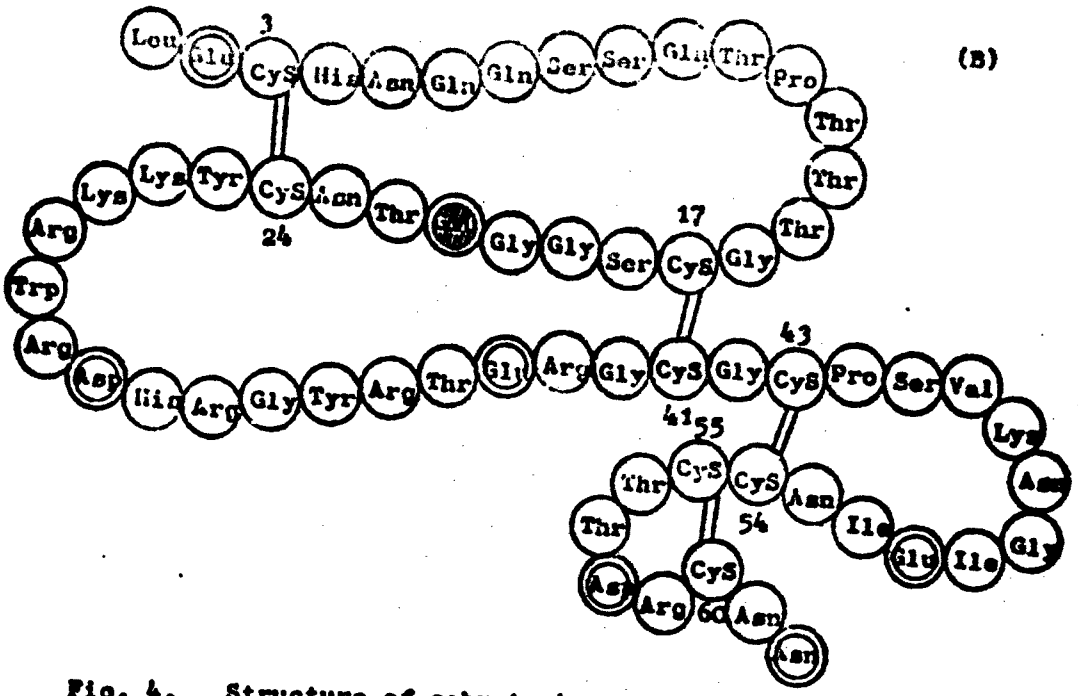


Fig. 4. Structure of cobrotoxin.
Two-dimensional schematic diagram showing the arrangement of the disulfide bonds and the sequence of the amino acid residues.

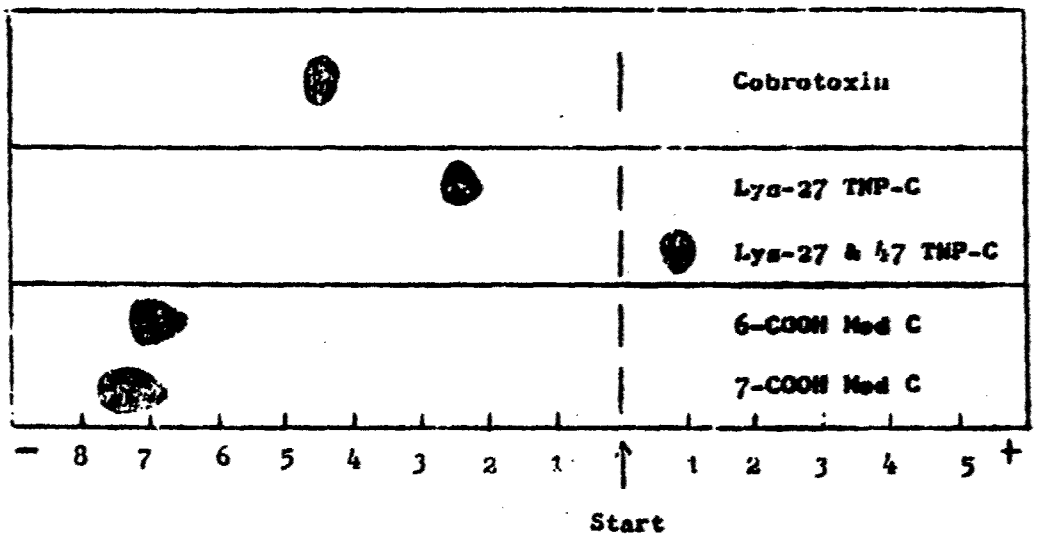


Fig. 5. Polyacrylamide gel electrophoretogram of cobrotoxin, TNP-cobrotoxin and carboxy-modified derivatives.

Electrophoresis was carried out in veronal buffer (pH 8.6, μ=0.05) at 250 V for 16 h at 4°. Lys-27 TNP-C, Lys-27 trinitrophenylated cobrotoxin; Lys-27 & 47 TNP-C, Lys-27 & 47 trinitrophenylated cobrotoxin; 6-COOH C, six carboxy-modified cobrotoxin; 7-COOH C, seven carboxy-modified cobrotoxin.

APPENDIX B-6

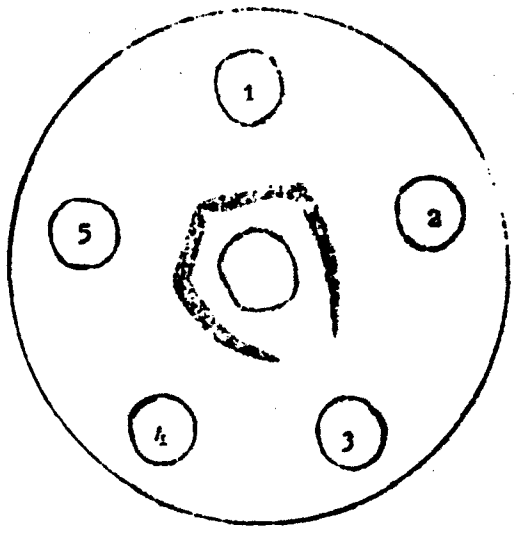


Fig. 6. Immunodiffusion in agar gel.

Central well: Anti-cobrotoxin sera.

Surrounding wells: (1) Cobrotoxin; (2) Lys-27 TNP-cobrotoxin; (3) Lys-27 & 47 TNP-cobrotoxin; (4) Guanidinated cobrotoxin; (5) TNP-guanidinated cobrotoxin.

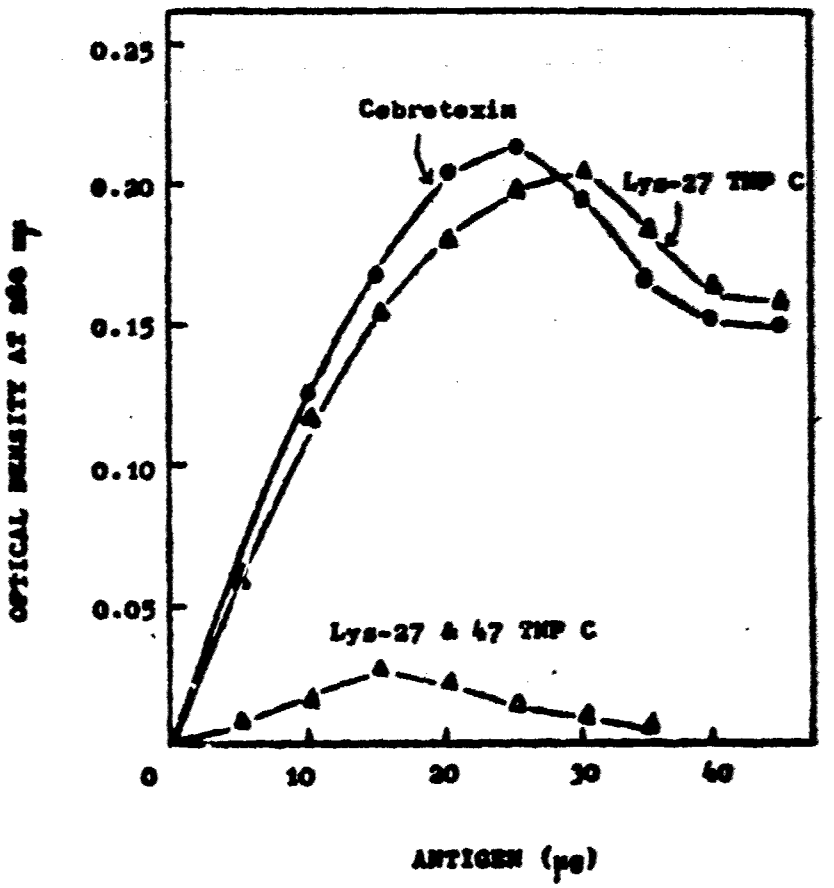


Fig. 7. Quantitative precipitin reactions of cobretoxin and its TNP-derivatives with anti-cobretoxin sera.

0.4 ml of antisera were used in each case of the analysis. ●——●, Cobrotoxin; ▲——▲, Lys-27 TNP-cobretoxin; ▲——▲, Lys-27 & 47 TNP-cobrotoxin.

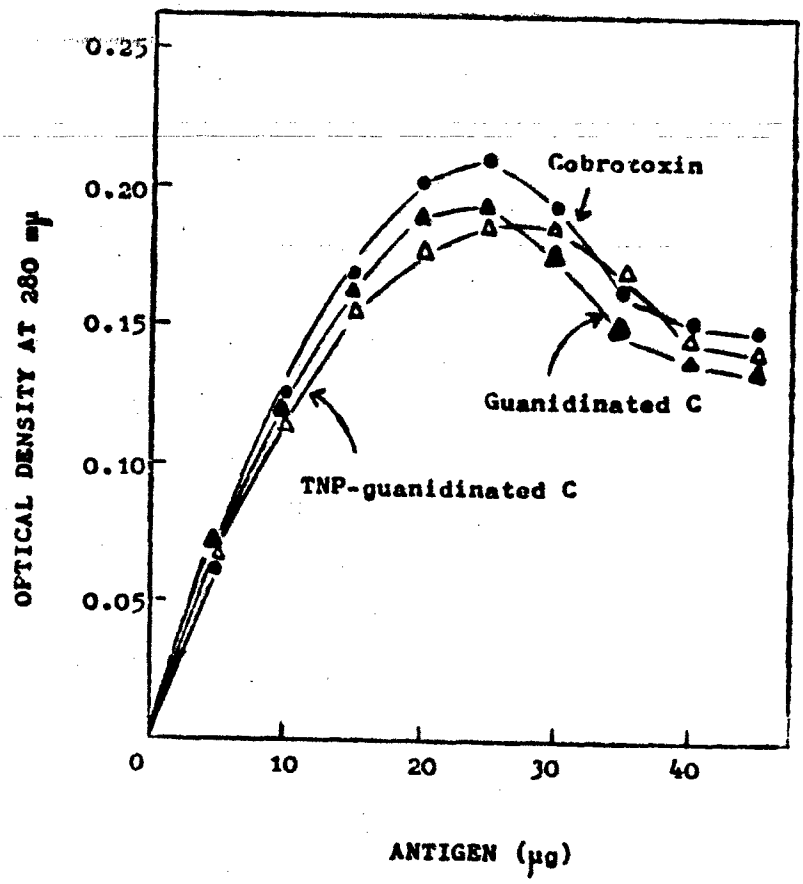


Fig. 8. Quantitative precipitin reactions of cobrotoxin, guanidinated cobrotoxin and TNP-guanidinated derivative with anti-cobrotoxin sera.

0.4 ml of antisera were used in each case of the analysis. ●——●, Cobrotoxin; ▲——▲, Guanidinated cobrotoxin; Δ——Δ, TNP-guanidinated cobrotoxin.

APPENDIX B-9

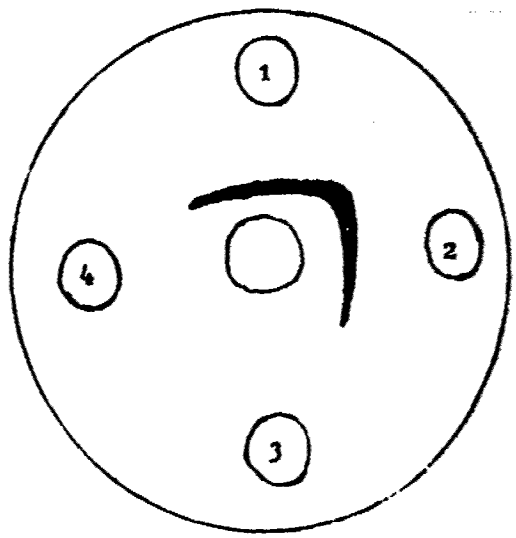


Fig. 9. Immunodiffusion in agar gel.

Central well: Anti-cobrotoxin sera.

Surrounding wells: (1) Cobrotoxin; (2) Six-carboxy-modified cobrotoxin; (3) Seven-carboxy-modified cobrotoxin; (4) 0.15 M NaCl.

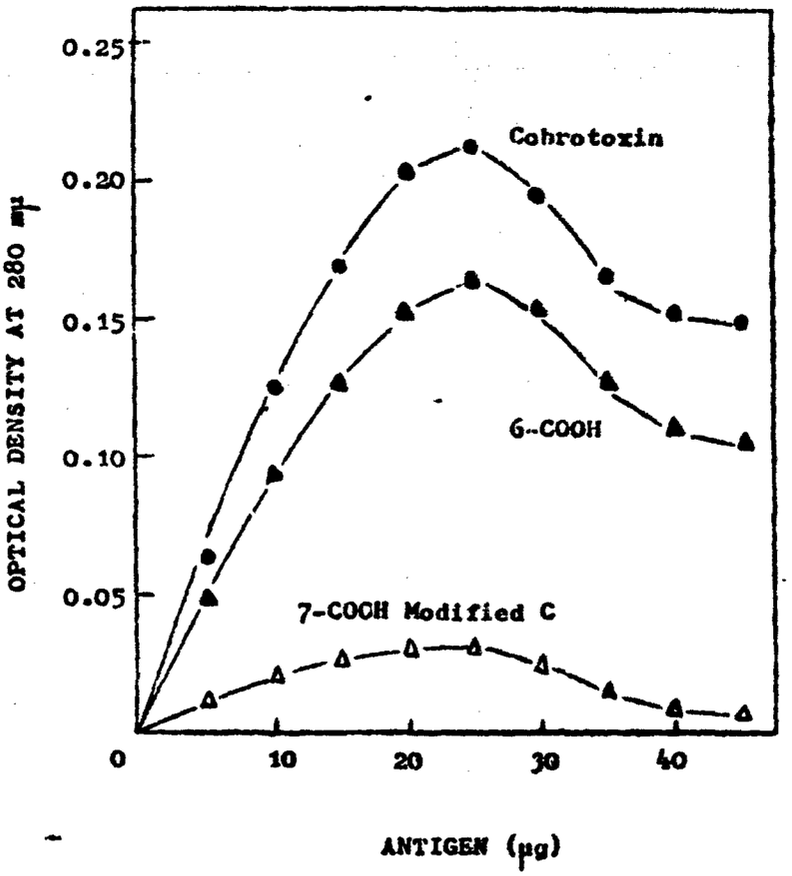


Fig. 10. Quantitative precipitin reactions of cobrotoxin and the carboxy-modified cobrotoxin with anti-cobrotoxin sera.

0.4 ml of the antisera were used in each case of the analysis. o——o, Cobrotoxin; Δ——Δ, Six carboxy-modified cobrotoxin; Δ——Δ, Seven carboxy-modified cobrotoxin.

List of Publications

1. The disulfide bonds of cobrotoxin and their relationship to lethality. Biochim. Biophys. Acta, 133 (1967) 366.

2. Optical retatory dispersion of cobrotoxin. J. Biochem., 61 (1967) 272.

3. Biochemical studies on the toxic nature of snake venom. Intern. Congr. Biochem., 7th, Tokyo Col. VIII, 1 (1967).

4. Studies on fluorescent cobrotoxin. Biochim. Biophys. Acta, 147 (1967) 600.

5. Study on I^{131} labeled cobrotoxin. Toxicon, 5 (1968) 295.

6. Optical retatory dispersion and circular dichroism of cobrotoxin. Biochim. Biophys. Acta, 168 (1968) 373.

7. Amino acid composition and end group analysis of cobrotoxin. Toxicon, 7 (1969) 43.

8. Immunochemical studies on cobrotoxin. J. Immun., 102 (1969) 1437.

9. The amino acid sequence of cobrotoxin. Biochim. Biophys. Acta, 188 (1969) 65.

10. Biochemical and immunochemical studies on cobrotoxin. The Snake, 2 (1970) 1.

11. Structure-activity relationships and immunochemical studies on cobrotoxin. Radiation Sensitivity of Toxins and Animal Poisons, IAEA-PL-334/6 (1970) 63.

12. The position of disulfide bonds in cobrotoxin. Biochim. Biophys. Acta, 214 (1970) 355.

13. Studies on the status of tyrosyl residues in cobrotoxin. Biochim. Biophys. Acta, 236 (1971) 164.

14. Studies on the status of free amino and carboxyl groups in cobrotoxin. Biochim. Biophys. Acta, in press (1971).

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